Detection and dissemination of the colistin resistance gene, *mcr-1*, from isolates and faecal samples in China

Xia Chen[1] , Xiaofei Zhao[1] , Jie Che[1] , Yanwen Xiong[1] , Yanmei Xu[1] , Lifeng Zhang[1] , Ruiting Lan[2] , Lining Xia[3] , Timothy R Walsh[4] , Jianguo Xu[1] , Jinxing Lu[1] , Juan Li[1]

[1] State Key Laboratory of Infectious Disease Prevention and Control, Collaborative Innovation Center for Diagnosis and Treatment of Infectious Disease, National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing, PR China

[2] School of Biotechnology and Biomolecular Sciences, University of New South Wales, Sydney, NSW 2052, Australia

[3] College of Veterinary Medicine, Xinjiang Agricultural University, Urumqi, PR China

[4] Department of Medical Microbiology, School of Medicine, Cardiff University, Cardiff, UK

ABSTRACT

Purpose

A recently identified colistin resistance gene, mcr-1, has been reported in many countries. In this study, we established a new real-time PCR method to detect it.

Methodology

We used a real-time PCR method to detect the mcr-1 gene in a variety of isolates and faecal samples from 20 provinces and municipal cities in China.

Results

Of the 2330 isolates (from 10 species) screened, 54 (2.3 %) isolates were positive for mcr-1. All of the mcr-1-positive isolates that were identified belonged to Escherichia coli strains, among which 9, 1, and 44 were identified as enteropathogenic E. coli, enteroadherent E. coli, and non-pathogenic E. coli, respectively. The majority of the mcr-1-positive isolates were obtained from farm animals from eight provinces and municipal cities across China. A total of 337 faecal samples, including 229 human and 108 pet animal faecal samples, were also screened for the mcr-1 gene. Of the 337 samples analyzed, six and eight human and pet animal faecal samples were positive for the mcr-1 gene, respectively.

Conclusion

The data demonstrate that the mcr-1 gene is highly prevalent in human and animal populations in China. This occurrence suggests that active surveillance of the mcr-1 gene is imperative in curtailing its spread

INTRODUCTION

Antimicrobial resistance is considered one of the most urgent public health problems of the twenty-first century. The phenomenon of antibiotic resistance threatens to affect human health worldwide. This problem has intensified following the emergence of carbapenem-resistant strains of *Enterobacteriaceae*, leaving colistin as one of the few remaining clinical treatment options for the treatment of infections which caused by carbapenem-resistant strains of Enterobacteriaceae. However, colistin is also widely used to treat infected animals, especially in China [1, 2].

In 2016, Liu and colleagues reported a plasmid-mediated colistin-resistance (designated *mcr-1*) gene in China. The *mcr-1* gene encodes a lipid A phosphoethanolamine transferase that is capable of inactivating colistin [1]. It has subsequently been found that the *mcr-1* gene is not only carried by different plasmids, but also located on the chromosome [1, 3–8]. Several MCR-1-positive *Enterobacteriaceae* have been isolated from both humans and animals in many different countries. These observations suggest that the *mcr-1* gene has been disseminated globally and is likely to impact both clinical and animal husbandry treatment strategies [9–13]. Thus, active surveillance pertaining to *mcr-1* gene dissemination is of paramount importance.

In this study, a novel real-time PCR assay was developed and used to detect the *mcr-1* gene in a variety of isolates from human and pet faecal samples in China.

METHODS

Isolates and faecal samples

A total of 2330 Gram-negative isolates were investigated in this study. These isolates from 20 provinces and municipal cities in China were obtained between 1998 and 2015. The species pertaining to these Gram-negative isolates are listed in Table 1 , and the geographical distribution details are presented in Figure 1 . Detailed information relating to isolate screening is shown in Table 2 . The number of isolates obtained during the years 2010 to 2015 (*n*=2014) was much more than the number of isolates obtained before 2010 (*n*=316). The pathogenic *Escherichia coli* isolates were identified by virulence gene PCR-typing and serotyping as done in a previous study [14]. Most of the isolates were collected from human faeces (from diarrhoea patients and healthy humans), poultry faeces (from broilers) and livestock faeces (from swine, cattle, goats and mutton sheep). A small proportion of the isolates were from commercially available food (from vegetables, chicken, pork, beef, meat stuffing, cured meat and animal innards), insects (from dung beetles and flies), fowl faeces (from egrets and pigeons), rodent intestinal contents (from pikas and marmots) and environmental samples (from fences and water in swine farms). The top three areas in respect of the number of isolates that were collected were Shandong, Henan and Beijing.

A total of 337 faecal samples were collected from Beijing and Hainan provinces between 2014 and 2015. Among these, 229 (159 and 70 samples from Beijing and Hainan, respectively) were obtained from humans and 108 (from a single Beijing animal hospital) were obtained from pet animals (77 from dogs and 31 from cats).

DNA extraction

DNA from bacterial isolates and standard strains was extracted using a commercial DNA extraction kit (TIANGEN TIANamp Bacteria DNA kit; TIANGEN BIOTECH). DNA from faecal samples was extracted using a commercial QIAamp stool extraction kit (QIAGEN). All of the extracted DNA templates were stored at −20 °C until further required.

Real-time PCR primers, fluorescence probe for *mcr-1* gene and real-time PCR assay

Real-time PCR primers and minor groove binder (MGB)-conjugated fluorescent probes were designed to amplify a 102 base pair DNA fragment (positions 143 to 244) from the *mcr-1* coding region (GenBank no. KP347127). The forward primer (5′-TCG GCT TTG TGC TGA CGA T-3′), the reverse primer (5′-AAA TCA ACA CAG GCT TTA GCA CAT A-3′) and the 6-carboxyfluorescein (FAM)-labelled *mcr-1*-specific probe (5′-FAM-CTG TCG TGC TCT TTG-MGB-3′) used in this study were procured from Shanghai GeneCore Biotechnologies. The LightCycler 480 II (Roche Diagnostics) was used for the amplification and detection of the *mcr-1* gene. Each PCR run included a negative control, an external *mcr-1*-positive control and an internal processing control (16S rDNA locus). The amplification conditions were 95 °C for 180 s followed by 40 quantitative cycles of 95 °C for 5 s, 58 °C for 20 s and 40 °C for 20 s.

Construction of the recombinant plasmid carrying the *mcr-1* gene

The *pEASY*-T5 Zero Cloning kit (TRANSGEN BIOTECH) was used to generate a plasmid harbouring the *mcr-1* gene-specific fragment (using the primers described above and a whole *mcr-1* gene-carrying plasmid) to measure copy number. The number of *mcr-1* gene plasmid copies was calculated using the value for the plasmid molecular weight and the following formula:

$$\text{Standard plasmid copies } \mu l^{-1}) = \frac{\text{Standard plasmid concentration } (\mu g\ \mu l^{-1}) \times \text{Avogadro's number} \times 10^{-9}}{660 \times \text{Number of recombinant plasmid base pairs}}$$

The PCR LightCycler 480 Probes Master kit (Roche Diagnostics) was used to generate standard plasmid serial dilutions, and nine concentration gradients from 1.0×10^{0} to 1.0×10^{8} copies μl^{-1} of standard plasmids were generated. Concentration gradient generation was performed in triplicate for standard curve construction and sensitivity evaluation.

Simulated faecal samples and *mcr-1* detection evaluation

An *E. coli* strain containing a whole *mcr-1* gene-carrying plasmid was used as a control and cultured overnight in brain–heart infusion broth until the culture reached an OD_{600} of 0.6. A plate-counting assay confirmed that the culture contained 2.2×10^{8} c.f.u. ml^{-1}. Nine concentration gradients from 1.1×10^{0} to 1.1×10^{8} c.f.u. ml^{-1} were generated by serial dilution. These dilutions were added to healthy human faeces to generate simulated *mcr-1*-positive faecal samples. DNA from faecal samples was extracted as described above.

Determination of specificity of the *mcr-1* PCR method

Escherichia coli strain ATCC 25922, *Acinetobacter lwoffi* strain ATCC 15309 and *Pseudomonas aeruginosa* strain ATCC 27853 do not harbour the*mcr-1* gene and were used as negative control strains. Genomic DNA extracted from these strains was used to evaluate the specificity of the*mcr-1* gene PCR method.

RESULTS

Sensitivity and specificity of the *mcr-1* gene PCR assay

A recombinant plasmid containing the *mcr-1* gene was serially diluted to generate nine concentration gradients (from 1.0×10^{0} to 1.0×10^{8}copies μl^{-1}) to determine the limit of detection by copy number. The detection limit of this *mcr-1* gene PCR method was determined to be 1.0×10^{1} copies per reaction, and a C_t value of less than 35 was considered as *mcr-1*-positive for the sample tested.

We next determined the detection limit for the assay by colony counting using the simulated faecal sample. This sample was diluted to between 1.1×10^{3} and 1.1×10^{8} c.f.u. ml^{-1} *mcr-1* positive *E. coli*. The limit of *mcr-1* detection by PCR was 1.1×10^{3} c.f.u. ml^{-1}, and a C_t value of less than 35 was considered as *mcr-1*-positive. We did not perform dilutions lower than 1.1×10^{3} c.f.u. ml^{-1}; thus, the detection limit may be even lower.

To evaluate the specificity of the assay, an *mcr-1* recombinant plasmid was used as positive control. The three negative control strains, *E. coli*ATCC 25922, *A. lwofii* ATCC 15309 and *P. aeruginosa* ATCC 27853, did not produce a positive signal.

Screening of *mcr-1* in a large collection of isolates from different species

A total of 2330 isolates were screened using the newly established PCR method. The isolates were obtained from 20 different geographical regions and consisted of both human and farm animal isolates (see Table 2). Fifty-four of the tested isolates (2.3 %) were positive for the *mcr-1* gene (see Table 3). Interestingly, all of the 54 *mcr-1*-positive strains were *E. coli* (54/1709, 3.2 %) including one (1/128, 0.8 %) enteroaggregative *E. coli* (EAEC) isolate, nine (9/402, 2.2 %) enteropathogenic *E. coli* (EPEC) isolates and 44 (44/697, 6.3 %) nonpathogenic *E. coli* isolates. The pathogenic *E. coli* isolates were

obtained from a variety of sources (diarrhoea patients, healthy human, commercially food, livestock or poultry), and the nonpathogenic *E. coli* isolates were from farm environment, livestock and poultry faeces (For details, see Table 3). The *mcr-1*-positive isolates were obtained from human faeces (3/1161, 0.3 %), livestock and poultry faeces (45/981, 4.6 %), food (4/103, 3.9 %) and environmental samples (2/9, 22.2 %) between 2010 and 2014. The oldest isolate was obtained from a faecal livestock sample in Xinjiang province in 2010, and the majority of *mcr-1*-positive isolates were from farm animals in Xinjiang (30 isolates, four from goat faeces in 2010, one from swine faeces in 2010, one from cattle faeces in 2012 and 24 from swine faeces in 2013) and Shandong province (12 isolates, all from poultry faeces).

Screening of *mcr-1* gene in human and pet animal faecal samples

A total of 337 faecal samples (229 from hospital inpatients and 108 from pet animals) were screened for *mcr-1*. Fourteen samples (4.2 %) were*mcr-1*-positive (Table 4). The positive rate was lower in inpatient samples than pet samples with six (2.6 %) positive inpatient faecal samples and eight (7.4 %) positive pet animal faecal samples identified, respectively. None of the 159 human faecal samples from Beijing was *mcr-1*-positive while six of the 70 (8.6 %) human faecal samples from Hainan were *mcr-1*-positive.

DISCUSSION

Sensitivity and specificity of the *mcr-1* PCR assay

The *mcr-1* gene was a new polymyxin resistance gene that was described last year [1]. In this study, a new PCR method was developed to detect *mcr-1*. The detection limit of the *mcr-1* gene, which was determined using a plasmid standard curve analysis and simulated faecal sample detection, was 1.0×10^{1} copies per reaction system and 1.1×10^{3} c.f.u. ml^{-1}, respectively. None of the *mcr-1* negative strains resulted in the generation of an amplification curve. Thus, the new *mcr-1* gene PCR method developed in this study displayed high sensitivity and specificity. This method is simple and rapid, and can be used for *mcr-1* detection in clinical, animal husbandry and food samples. From screening a large collection of isolates, human and pet animal faecal samples, we demonstrated that this PCR method is effective for direct detection of the *mcr-1* gene from faecal specimens.

Dissemination of the *mcr-1* gene throughout China

Liu and colleagues have reported that a plasmid-mediated colistin resistance gene, *mcr-1*, can be carried by *E. coli* and *Klebsiella pneumoniae*isolates from animals and inpatients in the south of China [1]. Our study expanded the geographic coverage by investigating whether *mcr-1*was present in isolates from 19 new provinces and municipal cities. This study also analysed a variety of sources including faeces from livestock, poultry, fowl, rodents and humans, along with food and environmental samples (see Figure 1 , Table 2). A total of 54 out of 2330 isolates and 14 out of 337 faecal samples were identified to be *mcr-1*-positive. We also observed that *mcr-1* isolates were present in eight ‘new’ regions of China including Xinjiang in the far northwest and Heilongjiang in the far northeast, suggesting that *mcr-1* is widespread in China (Figure 1). For the *mcr-1*-positive isolates detected in this study, we found that the *mcr-1*-carrying plasmids were different sizes varying from less than 60 to 150 kb (data not shown), suggesting transfer of the *mcr-1* gene between plasmids. Liu *et al.* [1] showed that the flanking sequences of the *mcr-1* gene were homologous in some of their isolates, also suggesting transfer of the *mcr-1* gene between plasmids. Other studies have shown that the *mcr-1* gene could be transmitted through conjugative plasmids [1, 3–6, 15]. Further studies will be needed to elucidate the mechanisms of the *mcr-1* gene.

Up until now, the *mcr-1* gene has predominantly been found in *Enterobacteriaceae* isolates [1, 2, 16–21], especially in *E. coli* strains. Moreover, all of the *mcr-1*-positive isolates from this study were *E. coli* strains. Importantly, 10 of the strains isolated between 2011 and 2014 were pathogenic *E. coli*. Three

of these pathogenic *E. coli* isolates (one EAEC strain and two EPEC strains) were obtained from humans, although colistin is not currently used in the routine treatment of *E. coli* infections. The presence of *mcr-1*-positive pathogenic *E. coli* isolates in infected patients highlights the potential threat to human health, with polymyxins previously representing 'the last line' of therapeutic agents to combat multidrug resistant Gram-negative infections.

Previous studies have shown that the prevalence of the *mcr-1* gene is lower in *E. coli* strains of human origin compared with strains isolated from animal and food samples [1, 10, 15, 18]. Our data are consistent with these findings. The *mcr-1*-positive rate in human isolates is 0.3 % (3/1144) while the rate in commercial food isolates and in livestock and poultry faecal isolates were 5.6 % (4/72) and 6.3 % (45/718), respectively. Overall our rate is lower than those reported by Liu and colleagues, in which the *mcr-1*-positive rate was 1.2 % in humans, 14.9 % in raw meat samples (pork and chicken), and 20.7 % in swine [1]. However, the difference in positive rates may be due to regional difference, sample type difference and difference in the years when the isolates were collected.

In our study, the *mcr-1*-positive isolates were collected between 2010 and 2014. Most of our *mcr-1*-positive strains were isolated in 2013 and the oldest isolate was collected from a Xinjiang province livestock faecal sample in 2010. However, the first observation of the *mcr-1* isolate in China occurred in the 1980s and rapid increase of *mcr-1* prevalence in *E. coli* from poultry was observed from 2011 onwards [2]. In the first report of *mcr-1* by Liu *et al.* [1], the positive rate was also higher in 2012–2014 isolates, than in 2011 isolates including isolates from pigs at slaughter house and chicken and pork meat. All data suggest that *mcr-1* prevalence has been rapidly increasing in recent years.

Direct detection and prevalence of the *mcr-1* gene in human and pet animal faecal samples in Beijing and Hainan

In this study, we detected the *mcr-1* gene in faecal samples using a PCR method and observed that six human and eight pet faecal samples were *mcr-1*-positive.

It has been suggested that pets can transmit *mcr-1 E. coli* [22]. To the best of our knowledge, our study is the first report to show that the*mcr-1* gene has been directly detected in pet faecal samples. Since pets are in direct and frequent contact with humans, it needs to be recognized that pets act as a reservoir for colistin-resistant bacteria and are potentially an important source of human infective colistin-resistant bacteria. All of the *mcr-1*-positive human faecal samples were from Hainan while none of the human faecal samples from Beijing was*mcr - 1*-positive, suggesting that there is a regional difference in its prevalence in the human population. However, it could also be due to sampling. Considering that the two EPEC isolates from food samples and eight pet faecal samples were *mcr-1*-positive from Beijing, it is likely that *mcr-1*-positive *E. coli* is present in the human population in Beijing though it has not been sampled in this study.

In conclusion, we utilized a newly established PCR method to screen a large collection of bacterial isolates from more than 10 species. We examined 2330 isolates of different species and found that only *E. coli* isolates carried the *mcr-1* gene. The *mcr-1*-positive rate was 2.3 % for all isolates and 3.2 % for *E. coli*. Screening of pet animal faecal samples from Beijing suggested an *mcr-1*-positive rate of 7.4 %. Thus, *mcr-1*-mediated resistance is not exclusive to humans and farm animals but also occurs in pet animals. Our study also extends the number of provinces and municipal cities, including Xinjiang, Hainan and Heilongjiang, in China where *mcr-1* dissemination has been reported. Our study highlights the importance of active surveillance in relation to the nationwide and global spread of *mcr-1*. Finally, to facilitate a more holistic surveillance strategy, the PCR detection method that was developed as part of this study should be employed to test for the presence of *mcr-1*in both isolates and faecal samples.

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Figure 1

Screening and detection of the *mcr-1* gene across China. Provinces/municipal cities where *mcr-1* screening was carried out and *mcr-1*-positive isolates were detected in this study. The areas in colour: provinces/municipal cities where isolates and/or faecal samples were collected. Pink: only isolates; yellow: isolates and faecal samples. Spots in colour represent areas where *mcr-1*-positive samples were isolated or detected. Blue spots: non-pathogenic *E. coli* strains; red spots: EPEC strains; yellow spots: EAEC strains; green spots: faecal samples. Grey bar represents *mcr-1* screening area previously described by Liu *et al*.

Table 1
Source and species of isolates used in this *mcr-1* screening study

pecies of isolates	Origin of isolates						No. of isolates
	Livestock faeces	Poultry faeces	Human faeces	Food	Other animals	Environment	
Acinetobacter spp.	0	0	104	0	0	0	**104**
Citrobacter spp.	0	0	11	0	0	0	**11**
E. coli							**1709**
Non-pathogenic *E. coli*	147	373	168	0	0	9	697
Pathogenic *E. coli**	238	34	561	103	76	0	1012
Enterobacter spp.	0	0	89	0	0	0	**89**
Klebsiella spp.	0	110	152	0	0	0	**262**
Proteus spp.	0	79	7	0	0	0	**86**
Pseudomonas spp.	0	0	15	0	0	0	**15**
Serratia spp.	0	0	26	0	0	0	**26**
Other isolate species	0	0	28	0	0	0	**28**
Total number	385	596	1161	103	76	9	**2330**

*Pathogenic *E. coli* contained 325 *E. coli* O157:H7 isolates, 157 diffusely adherent *E. coli* (DAEC) isolates, 128 enteroadherent *E. coli* (EAEC) isolates and 402 enteropathogenic *E. coli* (EPEC) isolates.

Table 2
Description of isolates in the *mcr-1* gene screening study

Area of collection	Year of collection	Origin of isolates								Total number of isolates
		Livestock faeces	Poultry faeces	Human faeces	Food	Insect	Fowl faeces	Rodent intestinal contents	Environment	
Anhui	1999–2014	19	8	23	–*	–	–	–	–	50
Beijing	2011–2014	–	–	327	30	–	1	–	–	358
Gansu	2015	–	–	44	–	–	–	–	–	44
Guangdong	2013–2014	–	–	103	–	–	–	–	–	103
Guizhou	2011–2012	–	–	3	–	–	–	–	–	3
Hainan	2011–2015	–	–	129	–	–	–	–	–	129
Hebei	2005, 2011	–	–	64	5	–	–	–	–	69
Heilongjiang	2009, 2012	3	–	–	–	–	–	–	–	3
Henan	2000–2015	61	–	363	6	1	–	–	–	431
Hubei	2006, 2011	–	–	20	2	–	–	–	–	22
Jiangsu	1999–2002	139	25	16	1	4	–	–	–	185
Ningxia	1998	2	–	–	–	–	–	–	–	2
Qinghai	2012–2015	–	–	6	–	–	–	54	–	60
Shandong	2012–2013	5	563	4	–	–	–	–	–	572
Shanghai	2013	–	–	2	–	–	–	–	–	2
Shanxi	2010	–	–	22	–	–	–	–	–	22
Sichuan	2012–2015	1	–	34	42	–	16	–	–	93
Tianjin	2001	5	–	–	–	–	–	–	–	5
Xinjiang	2010–2013	147	–	–	–	–	–	–	9	156
Yunnan	2001	3	–	1	17	–	–	–	–	21
Total	1998–2009	230	33	17	31	5	–	–	–	316
	2010–2015	155	563	1144	72	–	17	54	9	2014
	1998–2015	385	596	1161	103	5	17	54	9	2330

*No isolates or no faecal samples were collected.

Table 3
Description of *mcr-1*-positive isolates in this study

Area of collection	Year of collection	Species of positive isolates	No. of positive isolates	Origin of isolates
Anhui	2014	EPEC	1	Diarrhoea patient's faeces
Beijing	2014	EPEC	2	Commercially available chicken and minced beef
Guangdong	2014	EPEC	1	Healthy human faeces
Henan	2011	EAEC	1	Diarrhoea patient's faeces
Heilongjiang	2012	EPEC	2	Cattle faeces
Shandong	2013	Nonpathogenic *E. coli*	12	Broiler faeces
Sichuan	2014	EPEC	1	Broiler faeces
	2014	EPEC	2	Commercially available pork and chicken
Xinjiang	2010–2013	Nonpathogenic *E. coli*	30	Broiler faeces
	2013	Nonpathogenic *E. coli*	2	Fence and water in swine farm
Total number			54	

Table 4
Description of *mcr-1*-positive faecal samples in this study

Area of collection	**Year of collection**	**No. of positive samples**	**Origin of faecal samples**
Beijing	2015	0	Human
Beijing	2015	8	Pets
Hainan	2014	6	Human
Total number		14	